

EXHIBIT 2

High frequency of *BRAF* mutation in papillary thyroid carcinoma.

The RAF proteins are highly conserved serine-threonine protein kinases that have an important role in cell proliferation, differentiation and programmed cell death¹. The RAF proteins activate MEK (mitogen activated protein kinase kinase), which in turn activates the MAPK (mitogen activated protein kinase) pathway². Inappropriate and/or continuous activation of this pathway provides a potent promitogenic force resulting in abnormal proliferation and differentiation in many human cancers³.

Recently, Davies et al⁴ reported that *BRAF* is frequently mutated in a variety of human tumors, especially in malignant melanoma and colon carcinoma. The most common reported mutation was a missense T to A transversion at nucleotide 1796 (Val → Glu codon 599) observed in 80% of the mutant tumors. Moreover, functional analysis revealed that this transversion was the only detected mutation that caused constitutive activation of BRAF kinase activity independent of *RAS* activation by converting BRAF into a dominant transforming protein⁴. In this study we sought to determine the frequency of *BRAF* T1796A mutation and further elucidate the importance of this mutation in different primary human tumors.

We screened 476 primary tumors including 214 lung, 126 head and neck, 54 thyroid, 27 Bladder, 38 cervical and 17 prostate cancers for the *BRAF* T1796A mutation using PCR restriction enzyme analysis. Amplification of exon15 followed by restriction by the endonuclease *TspRI* identified the *BRAF* T1796A mutation. *TspRI* digestion of the PCR fragment yielded three major bands at 125, 87, and 12 bp in the wild type allele. The T1796A mutation abolished the restriction site resulting in a prominent 212 bp band from the mutant allele and residual bands from the normal allele (fig 1a). Reamplification

followed by direct manual sequencing in five cases validated the TspRI assay (fig 1b). As positive controls for the *BRAF* T1796A mutation we used cell lines HTB71, HTB72, A2058 and ME180, HCT116 as negative controls.

The *BRAF* T1796A mutation was identified in 22 of 37 (60%) papillary thyroid carcinomas (table 1), 6 of 126 (4.8%) head and neck cancers, and 4 of 214 (1.9%) lung cancers. Moreover, we analyzed 9 common thyroid cell lines (KAK1, KAT5, KAT7, KAT10, DRO, ARO, MRO 87-1, WR0-821, C643) and found the same *BRAF* mutation in 6 of the 9 lines (66%). We did not identify any mutations in bladder, cervical and prostate primary tumors. Furthermore no mutation was identified in biopsies from 17 benign thyroid conditions (nodular goiter, follicular adenoma, atypical follicular adenoma, adenomatous hyperplasia), 11 follicular thyroid carcinomas, 3 medullary thyroid carcinomas and 3 Hurthle cell carcinomas.

Papillary and follicular thyroid carcinomas originate from the thyroid follicular epithelial cells. To date, oncogenic mutations in *RAS* and *RET/PTC* rearrangements have been observed in follicular thyroid carcinoma and papillary thyroid carcinomas respectively^{5,6}. *RAS* mutations are common in follicular thyroid cancers reaching 50% in some studies, but were less common (5-20%) in papillary thyroid tumors⁵. Our observation of a high frequency of *BRAF* activating mutations in papillary thyroid carcinoma suggests that *BRAF* activation, and in turn activation of the RAF/MEK/MAPK signaling pathway, is a common and important biologic mechanism in the development of human papillary thyroid carcinoma. This observation is also consistent with the reported inverse association between the presence of *BRAF* and *RAS* mutations in other cancer types^{4,7}. *RASSF1A* contains a *RAS* association domain and suppresses tumor growth⁸.

Interestingly, we also observed a statistically significant inverse relationship of *BRAF* mutation with inactivation of the *RASSF1A* gene by promoter methylation in thyroid cancers ($P=0.016$, Fisher's exact test, data not shown).

The importance of the *RAS* pathway in thyroid cancers is further exemplified by the presence of common activation *RET* mutations in medullary thyroid tumors and their transforming effect through activation of the RAS/RAF/MEK pathway⁹. Moreover activation of the RAS/RAF/MEK/MAPK is known to induce genomic instability in thyroid PCCL-3 cells¹⁰ and inhibition of the MAP kinase pathway has led to decreased cellular proliferation of human thyroid cancer cell lines¹¹. All the above observations suggest that activation of the RAS/RAF/MEK/MAPK pathway at various points in the pathway is a key event in common thyroid tumors. Our results have identified *BRAF* T1796A mutation and subsequent activation of the RAF/MEK/MAPK signaling pathway as a common and important mechanism in the development of primary papillary thyroid carcinoma.

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Table 1: *BRAF* mutations in primary human tumors and thyroid cell lines.

Tumor type	No of samples screened	No of T1796A mutations	Per cent
Thyroid			
Papillary ca	37	22	60
Follicular ca	11	0	0
Hurthel ca	3	0	0
Medullary ca	3	0	0
Benign tumors	17	0	0
Thyroid cell lines*	9	6	66
Others			
Lung cancer**	214	4	1.85
Head & neck cancer***	126	6	4.8
Cervical cancer****	38	0	0
Prostate cancer	17	0	0
Bladder	27	0	0
Total	502	38	7.5

* Including KAK1, KAT5, KAT7, KAT10, DRO, ARO, MRO 87-1, WR0-821, C643

** Four out of 116 lung adenocarcinoma

*** Six out of 77 HNSCC (head and neck squamous cell carcinomas)

**** Including 22 squamous cell carcinomas and 16 adenocarcinomas of the uterine cervix.

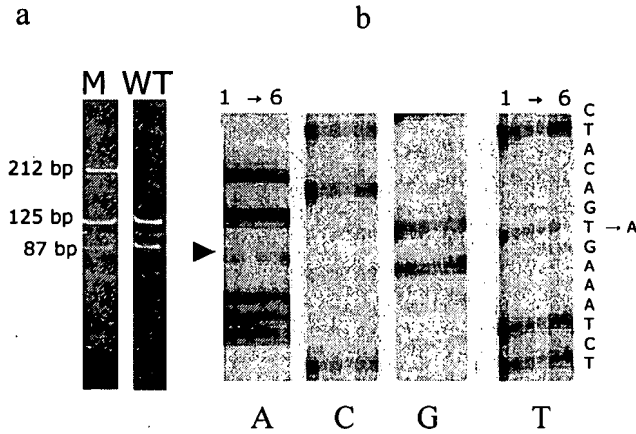


Figure 1: TspRI restriction enzyme analysis and exon 15 sequence analysis of *BRAF*.

Fig 1a: TspRI restriction enzyme analysis of exon 15 of *BRAF*; restriction pattern of T1796A mutation (M) and wild type (WT).

Fig 1b: DNA sequence of exon15 from papillary thyroid samples harboring the T1796A mutation (arrow head), cases T569, T203, T228 and T171 in lanes 1,2,4,5 respectively.

Lane 3 is a thyroid adenomatous hyperplasia (T530) negative for the T1796A mutation.

Lane 6 is the melanoma cell line HTB 72 which carries a homozygous T1796A mutation.